

Ex Vivo Expansion of Hematopoietic Stem Cells Using a Defined Culture Medium

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Abstract

Autologous or allogeneic hematopoietic stem cell (HSC) transplantation is a commonly used therapy for cancer patients following chemotherapy to reconstitute the ablated hematopoietic system. *Ex vivo* expansion of HSC is frequently exploited to overcome the limited availability of HSCs and progenitors for clinical applications and the understanding of hematopoiesis. The use of conventional basal cell culture medium supplemented with bovine serum often has resulted in unpredictable HSC expansion and presents potential exposure to adventitious viral agents (AVAs). For this reason, we have investigated the development of defined cell culture reagents capable of supporting optimal HSC *ex vivo* expansion and differentiation. A number of formulations were evaluated for their ability to support survival and expansion of CD34⁺ HSCs. Granulocyte colony stimulating factor (G-CSF)-mobilized HSCs obtained from healthy individuals (>95% CD34⁺) were used in this study. All cell culture media were supplemented with recombinant human cytokines Flt-3 ligand, stem cell factor (SCF) and thrombopoietin (TPO) in a 14-day *in vitro* expansion period. Several developed reagents supported cell expansion 100 - 300 fold after 14 days compared to 150 - 400 fold expansion supported by the currently commercially available media. In analyzing the expanded cultures in the test formulations, it was determined that the majority of cells had undergone differentiation into progenitors of the lineages of myeloid, lymphoid, erythroid and megakaryocyte. Only a small fraction of cells retained the CD34⁺ phenotype after 14 days. Of interest, one of the developed formulations was capable of expanding and maintaining the CD34⁺ population to levels comparable or greater than that of commercially available media. In conclusion, preliminary tests of a panel of formulations have confirmed that CD34⁺ HSCs can be expanded and maintained in a defined medium for up to 14 days, and extensive proliferation and expansion into hematopoietic lineages are achievable with administered cytokine cocktails.

Introduction

Hematopoietic stem and progenitor cells are frequently characterized by the surface expression of CD34. Down-regulation of CD34 has been shown to correlate with the loss of self-renewal property and reflect the differentiated fate of HSC in cell culture. Here, the growth and development of HSCs cultured in various serum-free media were monitored by flow cytometry of the CD34 expression. The overall expansion capacity of the medium was determined by the fold expansion of total nucleated cells (TNC) and of CD34⁺ cells obtained after 14 days in culture.

The benefits of using a serum-free medium for the expansion of HSCs include minimizing risk of AVAs in addition to minimizing performance effects due to lot-to-lot variability in serum. Cellular therapies do not utilize treatments for the reduction of AVAs prior to delivery, and while serum irradiation can significantly reduce AVAs, it cannot eliminate all risk. Serum contains known and unknown regulatory and growth factors which can affect and sometimes inhibit CD34⁺ proliferation. Therefore, a serum-free medium with defined components offers advantages over the use of a basal medium supplemented with serum. These considerations taken together strongly suggest that the use of serum-free media for the expansion of CD34⁺ populations is the preferred method for *ex vivo* expansion of cells.

Materials and Methods

Cells
Granulocyte colony stimulating factor (G-CSF)-mobilized CD34⁺ cells were obtained from Cell Therapy Core at the University of Minnesota Cancer Center (Minneapolis, Minnesota USA). The CD34 expression on these cells are consistently >95%.

Cell Culture Media
Control medium (StemSpan™ Serum-Free Expansion Medium, Catalog No. 09600) was purchased from StemCell Technologies (Vancouver, British Columbia CANADA). Cell culture test media were prepared by JRH Biosciences' imMEDIATE ADVANTAGE™ Program (Lenexa, Kansas USA).

Reagents
Lipoprotein, low density (LDL) (Product No. L7914) and holo-Transferrin human (Product No. T0665) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri USA); transferrin, human (holo) (Catalog No. 4455) was purchased from Serological Corporation (Norcross, Georgia USA). Recombinant cytokines including human SCF (rh-SCF), rhTPO and Flt-3 (Catalog No. SMPK-8), bovine serum albumin (BSA) and human holo-transferrin were provided by R&D Systems (Minneapolis, Minnesota USA).

Cell Culture
Cell culture was performed at R&D Systems. Briefly, 1 x 10⁶ cells were seeded per well in a 24-well tissue culture plate in 1 mL volume of medium containing 100 ng/mL of each of the following cytokines: rhSCF, rhTPO, rhFlt-3. LDL was supplemented where appropriate to 40 ng/mL just prior to use. Each culture condition was prepared in duplicates or triplicates. Cells were maintained in a 5% CO₂ humidified incubator at 37 C for a total of 14 days. On days four, eight, 12 or when extensive growth was observed in the wells, half of the medium was carefully removed and then replenished with the same volume of medium supplemented with cytokines and LDL. Additional medium was added to wells as necessary in order to maintain the cells in logarithmic phase growth. On day 14, cells from each well were counted on a hemacytometer to obtain the total nucleated cell (TNC) count.

Flow Cytometry
Fluorescence activated cell sorting (FACS) analysis of the expanded cell cultures was performed at R&D Systems. On day 14 after cell counting, duplicate samples were pooled, washed once with cold FACS buffer (2% Fetal Bovine Serum (FBS) and 0.1% sodium azide in PBS) and then resuspended in FACS buffer. CD34-conjugated phycoerythrin (PE) antibody (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, California USA) was added to 1 x 10⁶ cells in total reaction volumes of 100 µL according to the manufacturer's instructions. The samples were incubated for 30 minutes at 2 to 8 C in the dark, and excess antibody was removed by washing the cells with cold FACS buffer. Cells were then resuspended in 400 µL FACS buffer and analyzed by flow cytometry. Data was acquired using a FACS Calibur and analyzed using CellQuest software at the R&D Systems' facility.

Results and Discussion

A total of 26 different base formulations were screened. Results were calculated as the fold increases in TNC from day 0 to day 14 as well as the fold expansions of CD34⁺ staining cells. The fold increases in the TNC populations in the best two of the formulations tested are represented in Figure 1 as compared to the control medium; Figure 2 demonstrates these increases normalized to that in control medium. Of the 26 formulations screened, test formulation 2 performed the best and was selected for further optimization.

Through screening the initial base formulations, it was learned that both albumin and transferrin were critical for growth and expansion of CD34⁺ stem cells in serum-free media. In order to address these critical components, further optimization of the selected formulation included the screening of three lots of BSA and four lots of human holo-transferrin.

Results from screening BSA lots indicated that one lot of BSA did not support TNC expansion (BSA lot 3) and that two lots (BSA lots 1 and 2) supported expansion of TNC to levels 60% of control medium (Figure 3). However, BSA lot 2 supported CD34⁺ proliferation equivalent to that in control medium (Figure 4). Retention of the CD34⁺ phenotype was a desired characteristic of the medium under development, therefore BSA lot 2 was selected to use in the formulation being optimized.

Human holo-transferrin from four vendors, indicated as transferrin lots 1 - 4, was tested for TNC expansion as well as the ability to maintain the CD34⁺ phenotype in culture. Lots 2 and 3 supported less than 10% of normalized TNC expansion, while transferrin lots 1 and 4 supported ~50% of the normalized TNC expansion (Figure 5). These lots (1 and 4) were further tested for CD34⁺ expansion capability. Despite the somewhat lower TNC expansion demonstrated in test lots 1 and 4 as compared to control medium, expansion of the specific CD34⁺ cell populations in both of these test lots was equivalent to, or greater than, that in the control medium and dependent on the vendor of transferrin tested (Figure 6). The best combination of BSA and transferrin yielded 70% greater CD34⁺ cell populations as compared to controls. Because of the improved selectivity of the desired cell populations, faster *ex vivo* expansion capabilities are achievable using this optimized medium.

Conclusion

A serum-free medium formulation has been developed which supports the expansion of HSCs and, more importantly, maintains the CD34⁺ phenotype as well or better than the benchmark product. Optimal combinations of critical components, including BSA and transferrin, can yield populations of CD34⁺ cells up to 170% of that cultured in the benchmark product. This targeted expansion capability of the optimized medium will enable a faster scale-up of CD34⁺ cell populations for use in *ex vivo* therapies. Additionally, the use of a defined serum-free medium significantly reduces risk of AVAs for the patients undergoing these therapies, as compared to the use of a basal medium supplemented with serum.

Acknowledgements

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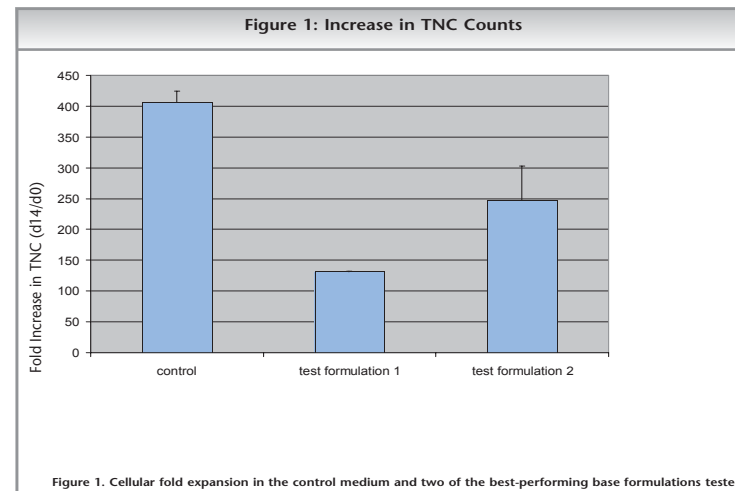


Figure 1. Cellular fold expansion in the control medium and two of the best-performing base formulations tested.

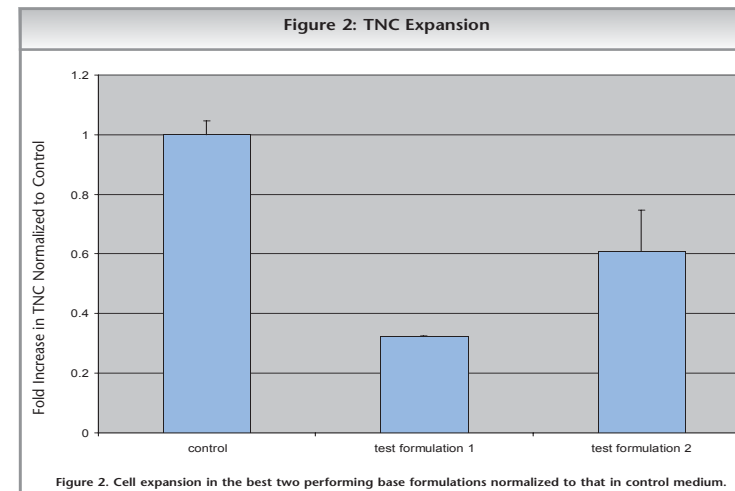


Figure 2. Cell expansion in the best two performing base formulations normalized to that in control medium.

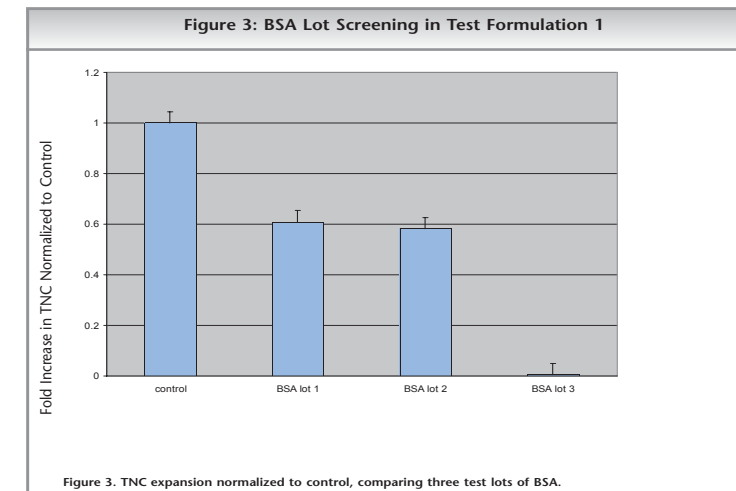


Figure 3. TNC expansion normalized to control, comparing three test lots of BSA.

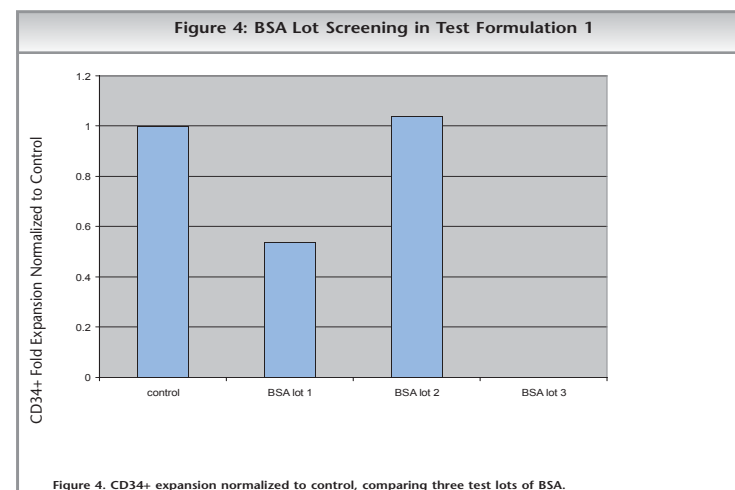


Figure 4. CD34+ expansion normalized to control, comparing three test lots of BSA.

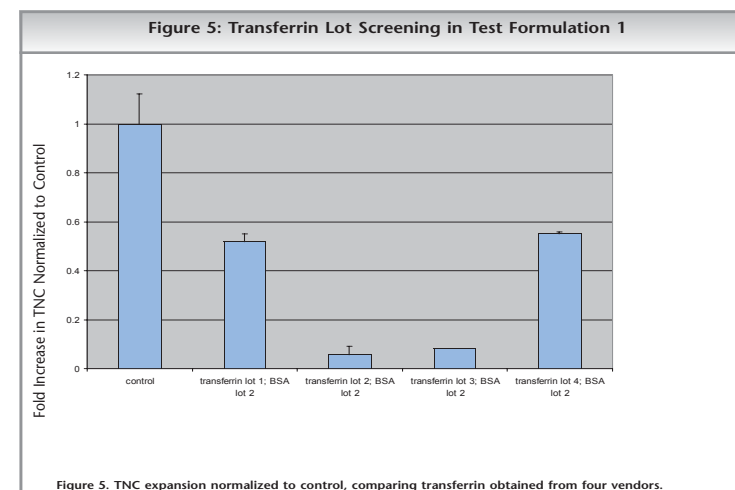


Figure 5. TNC expansion normalized to control, comparing transferrin obtained from four vendors.

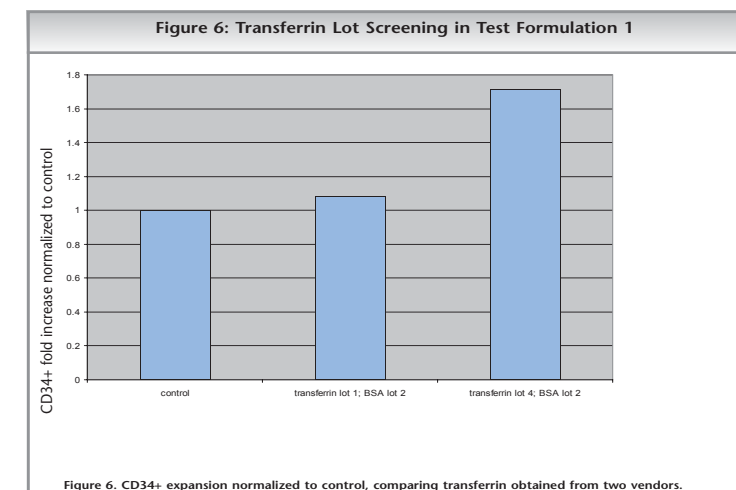


Figure 6. CD34+ expansion normalized to control, comparing transferrin obtained from two vendors.



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